Semaphorin 5B is a repellent cue for sensory afferents projecting into the developing spinal cord

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ABSTRACT

During vertebrate development, centrally projecting sensory axons of the dorsal root ganglia neurons first reach the embryonic spinal cord at the dorsolateral margin. Instead of immediately projecting into the grey matter, they bifurcate and extend rostrally and caudally to establish the longitudinal dorsal funiculus during a stereotyped waiting period of approximately 48 h. Collateral fibres then extend concurrently across multiple spinal segments and project to their appropriate targets within the grey matter. This rostrocaudal extension of sensory afferents is crucial for the intersegmental processing of information throughout the spinal cord. However, the precise cues that prevent premature entry during the waiting period remain to be identified. Here, we show that semaphorin 5B (Sema5B), a member of the semaphorin family of guidance molecules, is expressed in the chick spinal cord during this waiting period and dorsal funiculus formation. Sema5B expression is dynamic, with a reduction of expression apparent in the spinal cord concomitant with collateral extension. We show that Sema5B inhibits the growth of NGF-dependent sensory axons and that this effect is mediated in part through the cell adhesion molecule TAG-1. Knockdown of Sema5B in the spinal cord using RNA interference leads to the premature extension of cutaneous nociceptive axons into the dorsal horn grey matter. This suggests that Sema5B may be a key regulator of sensory axon entry into the developing spinal cord.

KEY WORDS: Sema5B, Dorsal root entry zone, Repulsion, Chick

INTRODUCTION

The vertebral dorsal root ganglion (DRG) contains a heterogeneous population of somatosensory neurons characterised by the sensory information they transmit (Eide and Glover, 1995). During spinal cord development, allafferent projections of DRG neurons initially respond to the same combination of cues that direct them into the dorsal root entry zone (DREZ), where their axial growth changes as they approach the dorsal horn grey matter (Davis et al., 1989; Masuda and Shiga, 2005). Here, they bifurcate and extend axons rostrally and caudally for numerous segments to form the dorsal funiculus and Lissauer’s tract during what is known as the ‘waiting period’, before simultaneously projecting collateral fibres into the grey matter across multiple segments (Davis et al., 1989; Eide and Glover, 1996; Schmidt et al., 2007). Despite their common origin and initial pathfinding into the DREZ, sensory collaterals establish connections in different regions of the grey matter depending on their sensory modality (Eide and Glover, 1997; Mendelson et al., 1992). Whereas sensory axons involved in nociception terminate in the dorsalmost layers and synapse with interneurons of the pain pathway, proprioceptive afferents project to the motoneurons located in the ventral horns (Eide and Glover, 1997).

Sensory axons reach the DREZ as discrete roots at each segment along the length of the spinal cord (Mauti et al., 2007). Preventing sensory axons from entering the grey matter and forcing their bifurcation and rostrocaudal extension is crucial in establishing intersegmental sensory circuits along the length of the spinal cord. This permits appropriate perception and reflexes (Sprague, 1958). Although we have learned much about the cues that regulate laminar targeting of sensory axons (Messersmith et al., 1995; Shepherd et al., 1997), the precise combination of inhibitory guidance cues that acts as a barrier against premature entry into the grey matter is still unclear.

One of the first identified proteins suggested to restrict sensory axon growth into the spinal cord was semaphorin 3A (Sema3A) (Messersmith et al., 1995). In mouse and chick, Sema3A is expressed in the spinal cord at the approximate time of sensory axon growth into the spinal cord (Adams et al., 1996; Fu et al., 2000; Masuda et al., 2003; Shepherd et al., 1996), and the phenotypes observed in Sema3a and neuropilin 1 (Nrp1; the Sema3A co-receptor) knockout mice (Behar et al., 1996; Gu et al., 2003; Kitsukawa et al., 1997) suggest that Sema3A might function as a barrier to premature axon entry into the spinal cord. Indeed, increasing the levels of Sema3A in the dorsal horn at the time of axon entry can prevent the entry of TrkA (also known as Ntrk1) positive cutaneous axons (Fu et al., 2000). However, evidence derived from spinal cord and DRG co-culture experiments and analysis of knockout mice suggests that at least one additional repulsive cue is expressed in the spinal cord and functions through the cell adhesion molecule TAG-1 [also known as axinon 1 or contactin 2 (Cntn2)] (Law et al., 2008; Masuda et al., 2003; Zuellig et al., 1992). Here we show that the semaphorin Sema5B is expressed early and throughout the spinal cord when axons first enter the DREZ. Also, we show that Sema5B is inhibitory to sensory axons and that knockdown of Sema5B in vivo results in early entry of TAG-1-expressing sensory axons into the spinal cord grey matter. This suggests that Sema5B may be a key regulator of sensory axon entry into the developing spinal cord.

RESULTS

Sema5B is expressed in the developing spinal cord

Using in situ hybridization, we found chick Sema5B to be expressed in various tissues of the embryonic nervous system, including the spinal cord, dorsal root ganglia, retina, tectum and olfactory epithelium (supplementary material Fig. S1). Similar to observations in the mouse (Adams et al., 1996), expression of Sema5B in the chick spinal...
cord is dynamic (Figs 1 and 2). At E3-3.5 (st21-23; Fig. 1A-D and Fig. 2A), Sema5B expression is observed broadly in the grey matter just as sensory axons are arriving at the DREZ. By E5 (st27), sensory axons have bifurcated and extended along the length of the spinal cord adjacent to the expression of Sema5B in the dorsal horn (Fig. 1E-H and Fig. 2B). At E6, Sema5B expression appears reduced along the periphery of the grey matter (Fig. 2C) and by E8 the expression of Sema5B has decreased throughout the grey matter, except in a population of cells in the ventral horn (Fig. 1LK and Fig. 2D). It has been well described that sensory collaterals do not enter the grey matter until E6 (st29) and then project to specific laminae targets by E9 (st35) according to their sensory modality (Eide and Glover, 1997; Mendelson et al., 1992; Perrin et al., 2001). The correlation between these times and the dynamic expression of Sema5B suggests the possible involvement of Sema5B in the timing and targeting of sensory collateral axons.

Sema5B acts as a repellent for chick sensory neurons in vitro

Previously, we showed that Sema5B can act as a repellent guidance cue for different populations of neurons during development (Browne et al., 2012; Lett et al., 2009; O’Connor et al., 2009; To et al., 2007). To test whether Sema5B can affect axon outgrowth of sensory neurons, dissociated DRG were obtained from chicks ranging from E5 to E8 and cultured on a confluent monolayer of HEK293 cells stably expressing Sema5B or a control vector. The growth of nerve growth factor (NGF)-responsive and neurotrophin 3 (NT-3)-responsive populations of sensory neurons was selected by the addition of either neurotrophin as previously described (Chan et al., 2008; Law et al., 2008). In all cultures with Sema5B-expressing cells, the mean axon length of sensory neurons at all ages examined was significantly shorter (by 30-40%) than observed in control cultures (Fig. 3A-D). Although it is possible that the transfection of Sema5B into HEK293 cells might have resulted in the expression of an additional unknown inhibitory protein, we have previously shown that purified Sema5B can function as an inhibitory factor and collapse DRG growth cones (Browne et al., 2012). Thus, exogenous Sema5B inhibits axon outgrowth of different classes of sensory neurons in vitro.

Nociceptive sensory axons invade the grey matter prematurely following knockdown of Sema5B

Having determined that Sema5B can act as a repellent cue for sensory axons in vitro, we examined the function of Sema5B in vivo by knocking down its expression using RNA interference (RNAi). Two short hairpin RNA (shRNA) sequences were validated by their ability to reduce Sema5B expression when transfected into HEK293 cells stably expressing HA-tagged Sema5B (Fig. 4). GFP expression showed control (empty pLL vector) and shRNA-positive cells, and Sema5B expression was determined by HA labelling (Fig. 4). Compared with the control (Fig. 4A-C), GFP-positive cells transfected with shRNA plasmids exhibited a substantial reduction in HA-Sema5B expression, confirming the effectiveness of the RNAi knockdown (arrows in 4D-I). Similarly, western analysis of lysates of shRNA-transfected HEK293 cells stably expressing Sema5B showed a considerable reduction in HA-Sema5B expression compared with control transfected cells (Fig. 4J). Because the transfection efficiency with the shRNA plasmids was not 100%, some HA-Sema5B remained detectable in the cell lysates, but a significant reduction was observed for each of the shRNA constructs (Fig. 4J, compare lanes 2-4 with lane 1). Furthermore, the knockdown effect of the two shRNA constructs combined was as effective as when the two constructs were transfected individually (Fig. 4J, lane 4 compared with lanes 2 and 3); therefore, the two constructs were also used in combination (each at half the concentration of single transfection) for knockdown experiments in vivo. By contrast, the same shRNA vectors did not reduce the expression of HA-tagged mouse Sema5B (Fig. 4K, compare lanes 2-4 with lane 1), confirming their specificity to the chick homologue. Thus, the two shRNAs significantly knocked down chick Sema5B overexpression 24 h after transfection and they did not knockdown mouse Sema5B.
Fig. 2. The expression of Sema5b mRNA in the developing spinal cord is dynamic. (A-D) In situ hybridisation of Sema5b mRNA in the chick spinal cord at the indicated stages. (E,F) In situ hybridisation results for sense strand cRNA probe. Sema5b expression at E3 (arrows in A; note that the dorsal spinal cord tissue is disrupted during processing) and at E5 (arrows in B) is strong throughout the grey matter. Longitudinal axon tracts established by sensory axons arriving at the DREZ can be distinguished at older ages (arrowheads in B,C,D). At E6, Sema5b is highest along the ventricular zone and the ventral horn (double arrows in C) and decreases in the rest of the grey matter (arrow in C). Sema5b expression is only present at high levels in the ventral horn at E8 (arrows in D). Scale bars: 100 μm.

To examine whether Sema5B can function as a barrier in the grey matter to prevent the premature entry of sensory axons, we knocked down its expression by electroporating shRNAs into the spinal cord at E3.5 (st21), just after primary sensory axons first reach the DREZ (Eide and Glover, 1995; Mendelson et al., 1992; Perrin et al., 2001). Embryos were sacrificed at E6 (st29), when collaterals normally begin to invade the grey matter (Eide and Glover, 1995; Mendelson et al., 1992; Perrin et al., 2001). Sensory axons were labelled with anti-TAG-1 to examine the timing and extent of their entry into the grey matter (Law et al., 2008). Control transfected spinal cords showed no TAG-1-labelled sensory axons inside the grey matter on either side of the spinal cord (Fig. 5A-C,G). By contrast, when Sema5B expression was knocked down, TAG-1-positive afferents showed a striking change in their pathfinding pattern after reaching the DREZ, as a significant number of axons prematurely invaded the grey matter (Fig. 5D-G). Furthermore, these early penetrating nociceptive axons did not appear to pathfind correctly, as the majority extended beyond their normal sites of termination and reached the ventricular zone at the midline of the spinal cord (Fig. 5E,F).

A possible explanation of this phenotype is that, in animals with reduced Sema5B expression, sensory axons are not being forced to turn after reaching the spinal cord from the dorsal roots. If this is correct then it would be expected that the majority of early penetrating axons would be located at sites of dorsal root entry. To examine this, we analysed the position along the rostral-caudal axis at which the sensory axons prematurely entered into the grey matter of Sema5B knockdown animals. We compared the number of aberrant fibres found in sections in which the dorsal roots enter the spinal cord (root sections, Fig. 5H) with the number of fibres in sections between dorsal root entry sites (non-root sections, Fig. 5H). In all experimental spinal cords analysed, significantly more aberrant projections were found on root sections compared with those found on non-root sections (Fig. 5I). Furthermore, aberrant projections were observed in 88% of root sections, whereas only 15% of non-root sections showed a phenotype. To examine this further, we DiI labelled a subset of peripheral DRG axons and examined their central projections at the dorsal roots in the spinal cords of Sema5B knockdown animals. Although the majority of axons turned and extended along the dorsal funiculus (Fig. 5J, arrows), a significant number of axons had extended into the grey matter (Fig. 5J,K). We could not follow the path of a single aberrantly projecting axon in the DREZ due to the intense DiI labelling of axons, but many of the single axons that entered the grey matter appeared to extend straight into the grey matter (Fig. 5K, arrows). Collectively, these data suggest that when axons first enter the DREZ they are normally inhibited from growing into the spinal cord by Sema5B and instead extend collaterals along the rostral and caudal length of the spinal cord.

Prooptroceptive axons do not invade the grey matter prematurely after knockdown of Sema5B

To examine whether Sema5B also acts as a barrier for other sensory afferents, we co-transfected HA-tagged mouse Sema5B (m5B) with the shRNA constructs. Co-transfection of HA-m5B and shRNAs prevented the premature entry of sensory fibres, demonstrating that the effects observed following shRNA transfection were specific to the knockdown of Sema5B (Fig. 5G). However, no rescue effect was observed when the shRNA constructs to chick Sema5B (c5B-KD) were co-transfected with an empty pDisplay (pDis) vector (Fig. 5G). These data strongly support the finding that Sema5B regulates sensory axon entry into the spinal cord grey matter.

Nociceptive afferents exhibit pathfinding errors following Sema5B knockdown

Next, we asked whether, in addition to acting as a barrier to early axon entry, Sema5B also regulates collateral fibre targeting of nociceptive axons once they have entered the spinal cord. We knocked down the level of Sema5B expression in the spinal cord at E5.5 (st27) by shRNA electroporation and examined the effect on nociceptive axon guidance...
at E8 (st33), when they normally establish contacts with their correct targets. In control spinal cords, TAG-1-positive collaterals extending from the dorsal funiculus remain confined to the dorsal lateral region of the dorsal horn (arrowheads, Fig. 7A-C). By contrast, after Sema5B knockdown, TAG-1-positive collaterals extend more ventromedially and target the ventricular zone surrounding the central canal (arrows, Fig. 7D-F), similar to our observations following Sema5B knockdown at earlier stages. We observed a significantly greater number of mistargeted nociceptive axons in Sema5B knockdown spinal cord (67±9 aberrant projections per 300 µm of spinal cord) compared with control spinal cord (20±3 aberrant projections per 300 µm; unpaired t-test, *P<0.05; n=5 animals for each condition). These findings suggest that Sema5B within the grey matter might also be important for guiding the nociceptive collateral afferents to their correct targets in the dorsal horn laminae.

To investigate whether the early entry of axons or aberrant pathfinding was due to abnormal development of the neural tube following electroporation, the cellular patterning in the grey matter and the expression of various transcription factors were analysed in control and knockdown embryos that had been electroporated at E3.5 and fixed at E6 (Fig. 8). Upon comparing control (Fig. 8A,C,E,G,I) and Sema5B knockdown (Fig. 8B,D,F,H,J) spinal cords, no difference in cell density or patterning (Fig. 8C,D) was observed, nor between electroporated versus non-electroporated sides of the spinal cord. Similarly, no difference in the expression of Isl1 (Fig. 8E,F), Nkx2.2 (Fig. 8G,H) or Pax6 (Fig. 8I,J) was observed. These results indicate that the patterning of the neural tube is not affected by electroporation in general, nor by the knockdown of Sema5B specifically.

Sema5B functions through TAG-1

Considerable evidence from in vitro and in vivo experiments has demonstrated the presence of a spinal cord-derived chemorepellent that functions in part though the immunoglobulin superfamily cell adhesion molecule TAG-1 (Law et al., 2008; Masuda and Shiga, 2005; Perrin et al., 2001; Sharma and Frank, 1998). Thus, we tested the possibility that Sema5B may function through the TAG-1 protein. We employed the same dissociated DRG overlay assay used above but added a monoclonal mouse anti-TAG-1 antibody to some cultures to inhibit the function of TAG-1 on sensory axons as described previously (Law et al., 2008).

We first show that the outgrowth of E4 sensory neurons cultured in the presence of NGF or NT-3 was inhibited by Sema5B (Fig. 9A). At this stage sensory neurons are just beginning to discriminate between growth factors for survival (Lefcort et al., 1996), and all of their axons express TAG-1 (Perrin et al., 2001). Surprisingly, in the presence of TAG-1 antibodies only neurons cultured in the presence of NGF showed a reduction in inhibition by Sema5B (Fig. 9A). This suggests that a subpopulation of neurons might be differentiating into NGF-dependent neurons and that their sensitivity to Sema5B is mediated through TAG-1. At E6, Sema5B was inhibitory for both populations of neurons (Fig. 9B), although at this stage NT-3-dependent neurons no longer express TAG-1 (Perrin et al., 2001). This suggests that the inhibitory effects of Sema5B are not mediated via TAG-1 for proprioceptive NT-3-dependent neurons. Indeed, whereas the addition of a function-blocking anti-TAG-1 antibody to the cultures blocked the inhibitory effect of Sema5B on NGF-dependent neuron outgrowth, it did not affect NT-3-dependent axons (Fig. 9). It is also interesting to note that the effects of the TAG-1 antibody were more pronounced at E6 when NGF-dependent neurons are more differentiated and rely specifically on NGF for survival. To examine whether the TAG-1 antibody may be overcoming the Sema5B inhibition by directly stimulating neurite outgrowth, we also cultured E6 neurons on control cells in the presence of the antibody. The antibody had no effect on neurite outgrowth of either population of neurons relative to the control and to each other (Fig. 9B).

These findings demonstrate that acutely blocking TAG-1 function is sufficient to eliminate the inhibitory effect of Sema5B on TAG-1-positive nociceptive axons and support the hypothesis that Sema5B acts partly through TAG-1 to inhibit the outgrowth of these axons. By contrast, the repulsive effect of Sema5B on NT-3-dependent neurons was not inhibited by the addition of an anti-TAG-1 antibody, suggesting the presence of a non-TAG-1 receptor complex on these neurons that mediates the repulsive effect of Sema5B.

DISCUSSION

Neurons are guided by a combination of inhibitory and attractive cues as they establish precise and complex circuits. A goal of the present study was to identify inhibitory cues that prevent sensory axons from prematurely entering the spinal cord grey matter. As sensory axons extend into the spinal cord as fasciculated dorsal roots (Mauti et al., 2007), it is important that axons are forced to bifurcate and extend along the length of the spinal cord before entering the grey matter to form intersegmental neural circuits. It is most likely that this mechanism has evolved to maximise circuit formation along the entire length of the spinal cord, particularly
between the regions where dorsal roots enter the spinal cord. We propose that Sema5B is a crucial contributor to this process.

In the present study we have shown that DRG neurons do not extend axons into the dorsal horn when Sema5B expression is high. In vitro assays show that sensory neuron outgrowth is inhibited by Sema5B over the embryonic time period (E5-E8) when sensory axons are extending into the spinal cord. Furthermore, functional analyses in vivo showed that Sema5B knockdown results in the premature entry of nociceptive TAG-1-expressing axons, particularly at the levels of dorsal root entry. Finally, we found that the adhesion molecule TAG-1 may play a role in the axonal responses to Sema5B. This is the first evidence of the involvement of Sema5B in sensory neuron circuit formation in the developing spinal cord and suggests that it plays a crucial barrier function that ensures uniform connectivity along the spinal cord (Fig. 10). It is important to note, however, that although proprioceptive axon outgrowth is inhibited by Sema5B, this inhibition does not appear to be mediated through TAG-1, and knockdown of Sema5B in vivo did not result in their premature entry into the spinal cord. This suggests that Sema5B might play a more restricted role in the regulation of proprioceptive axon entry into the spinal cord grey matter.

**Sema5B is a functional barrier to sensory axons**

We found that Sema5B is present in the chick spinal cord as early as E3, the developmental period when the first sensory axons are targeting the DREZ. Our in vivo analysis suggests that Sema5B acts as a barrier at the border of the dorsal grey matter to the DRG axons that have reached the DREZ. This repulsion must be significant as it forces the growth cones to turn ~90° in both rostral and caudal directions. At this time it is vital that sensory axons extend along the length of the spinal cord in order to form the nerve tracts in the dorsal white matter before extending collaterals into the grey matter. This facilitates the integration of sensory information across multiple segments along the rostrocaudal axis of the spinal cord. When the Sema5B barrier is removed or reduced, axons enter the grey matter prematurely at the dorsal roots and appear to extend straight into the grey matter. This barrier function of Sema5B is similar to its function in preventing corticofugal fibres from aberrantly projecting into the ventricular zone (Lett et al., 2009) as well as to its function in confining neurites of multiple neuron types to their appropriate lamina in the retina (Matsuoka et al., 2011).

It is surprising that proprioceptive axons did not extend prematurely into the grey matter after Sema5B knockdown. These axons are responsive to Sema5B in vitro and show a similar reduction of neurite outgrowth at the same embryonic ages as the nociceptive fibres. Presumably, the proprioceptive fibres are inhibited in vivo by a combination of cues, including Sema5B, and the reduction of any one of these cues might not be sufficient to allow early entry into the spinal cord grey matter.

It has previously been suggested that other inhibitory cues are required for the correct pathfinding of sensory afferent axons. For example, Sema3A is expressed in the spinal cord and is a repellent cue to DRG axons (Messersmith et al., 1995; Shepherd et al., 1997). A number of reports have shown that Sema3A is expressed in the spinal cord at the time that sensory axons first reach the DREZ in chick and mouse (Adams et al., 1996; Fu et al., 2000; Masuda et al., 2003; Wright et al., 1995). In animals lacking Sema3A or Nrp1, only a few aberrant sensory projections into the central nervous system were observed (Kitsukawa et al., 1997; Taniguchi et al., 1997), although these reports were not examining early entry into the spinal cord specifically, and therefore might have underestimated this phenotype. Indeed, when the Sema-binding domain of Nrp1 was mutated, TrkA-positive fibres were observed to prematurely enter the spinal cord grey matter (Gu et al., 2003). Similarly, increasing the levels of Sema3A in the dorsal horn grey matter at the time of normal ingrowth can prevent the entry of TrkA-positive axons (Fu et al., 2000). Thus, these results suggest that additional semaphorins, in particular Sema3A, contribute to the barrier function.

Watanabe et al. (2006) have suggested that the brief appearance of netrin 1 in the mouse dorsal spinal cord (between E12.5 and E13.5) acts as an inhibitory cue to prevent axons from entering the mantle layer during the waiting period (Watanabe et al., 2006). However, as the authors pointed out, the upregulation of netrin 1 occurs midway through the waiting period, whereas the arrest of axial axon trajectory occurs from E10.5, when axons reach the DREZ. This means that there must be other molecules inhibiting the invasion of axons. Furthermore, netrin 1 expression is restricted to the floor plate in chick throughout sensory circuit development, which does not support its role as a barrier (Guan and Condic, 2003; Wang et al., 1999). Another molecule that has been proposed to function as a barrier between the central nervous system and the peripheral nervous system is Sema5A (Mauti et al., 2007). Mauti et al. (2007) showed that Sema5A is expressed by boundary cap cells near both the dorsal and ventral root entry sites in early development.
embryogenesis (E3) and the downregulation of Sema6A leads to the disorganisation of dorsal roots. The authors did not show, however, whether the subsequent timing or patterning of sensory afferents was changed; thus, further studies are required to fully elucidate the function of Sema6A in this light.

**Sema5B functions through TAG-1**

Cell adhesion molecules, including TAG-1, have long been known to function during axon guidance in processes such as fasciculation and outgrowth (Furley et al., 1990; Zuellig et al., 1992). Only recently have these molecules received attention as binding partners to mediate the responses to repulsive guidance cues in the nervous system (Law et al., 2008). TAG-1 is linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (Furley et al., 1990; Zuellig et al., 1992) and can bind homophilically to other TAG-1 molecules on adjacent cells (Freigang et al., 2000; Rader et al., 1993). Perrin et al. (2001) showed that TAG-1 is expressed by all cell bodies and axons of DRG neurons during early stages of development but its expression then becomes restricted to the NGF-dependent, TrkA-expressing nociceptive fibres after E6 (Perrin et al., 2001; Snider and Silos-Santiago, 1996). This temporal correlation provides support for TAG-1 as a component of the receptor complex for mediating the inhibitory effect of Sema5B on nociceptive sensory fibres.

Additional work by Perrin et al. (2001) has shown that TAG-1 is required for correct nociceptive cutaneous axon targeting in the dorsal region of the spinal cord (see Fig. 10). They found that, after injection of function-blocking antibodies against TAG-1 into the cerebral aqueduct of the developing spinal cord, nociceptive axons projected aberrantly into the dorsal horn (Perrin et al., 2001). Strikingly, the phenotype observed when TAG-1 function is perturbed is extremely similar to the phenotypes observed when Sema5B was knocked down in the developing spinal cord. Specifically, nociceptive axons projected prematurely into the dorsal grey matter (1 day before the normal time of collateral formation) and grew aberrantly toward the midline, dorsal to the central canal (the future lamina III region), instead of innervating laminae I and II as seen in normal animals. By contrast, the proprioceptive fibres were not affected by the function-blocking TAG-1 antibodies (Perrin et al., 2001). Law et al. (2008) examined the role of TAG-1 in regulating sensory axon responses to diffusible guidance cues in mice by following the pathways taken by sensory afferents in TAG-1 (-/−) null mice. Similar to observations in the chick, they first showed that TAG-1 is
expressed in all DRG neurons until the time of axon arrival at the DREZ (E10.5 in mice), but, by E12.5, TAG-1 expression became restricted to TrkA-expressing nociceptive fibres, 1 day before they extended collaterals into the dorsal horn. Law et al. (2008) also saw similar phenotypes in the TAG-1 null mice as were observed in our Sema5B knockdown experiments and which were also observed after TAG-1 antibody injections as discussed above (Perrin et al., 2001). They observed premature projections of cutaneous axons in TAG-1 null mice, particularly focused around points of dorsal root entry (Fig. 10). These authors argued that TAG-1 is required on sensory axons to mediate their response to a non-Sema3A diffusible repellent guidance cue(s) found in the spinal cord, although they had not identified the specific cue(s) (Law et al., 2008).

Presently, the mechanism of TAG-1 function in Sema5B signalling is unknown. Dang et al. (2012) have recently shown that TAG-1 regulates Sema3A signalling by differential endocytotic trafficking of components of the Sema3A receptor complex (Dang et al., 2012). Whether TAG-1 functions in a similar way for Sema5B signalling is not known. Additional potential receptors for Sema5B have also been described recently. Using a combination of Sema5b and plexin A1 and A3 null mouse lines, Matsuoaka et al. (2011) have shown that Sema5B signals in part through plexin A1 and/or A3 to regulate retinal lamination. Whether plexin A1 and A3 function with a co-receptor such as Nrp1/2 or TAG-1 is unknown.

MATERIALS AND METHODS

Animals
Fertilised White Leghorn chicken eggs were obtained from the University of Alberta and incubated at 38°C. Embryos were staged according to Hamburger and Hamilton (1951).

In situ hybridisation
Chick embryos younger than E7 were fixed in 4% (v/v) paraformaldehyde (PFA; Sigma) in diethylpyrocarbonate (DEPC)-treated PBS at 4°C for 8 h followed by washing in PBS. Chicks at E7 or older were first fixed via pericardial infusion by injecting PBS into the heart for 2 min followed by 4% PFA for 10 min. Embryos were placed in 30% sucrose in DEPC-treated PBS overnight at 4°C for cryoprotection. RNA probes were prepared using a digoxigenin labelling kit and employed as described by the manufacturer (Roche Molecular Biochemicals). Antisense digoxigenin-labelled probes were generated from the C-terminus of Sema5B. Sense probes generated from the same region were used as a control.

Neurite outgrowth assay
DRG were dissected from E4, E5, E6, E7 and E8 chick embryos into cold DMEM (Invitrogen) and dissociated in 0.25% (v/v) trypsin-EDTA (Invitrogen) as previously described (Browne et al., 2012). Neurons (8×10^5 cells) were seeded on top of a confluent layer of stable HEK293 cells expressing either an empty pDisplay vector (control) or an HA-tagged chick Sema5B (chick HA-Sema5B), similar to as previously described (Matsuoka et al., 2011). To select for the growth of nociceptive neurons, culture medium was supplemented with 40 ng/ml 7S nerve growth factor (NGF; Invitrogen), and to select for the growth of proprioceptive neurons the same amount of neurotrophin 3 (NT-3, also known as Ntf3; PeproTech) was used (Chan et al., 2008; Law et al., 2008; Messersmith et al., 1995). Primary antibody incubations were performed with mouse anti-Tuj1 (also known as Tubb3; 1:500, Sigma, T8578) for visualisation of sensory neurons and
rabbit anti-HA (1:500, Cell Signaling, #3724) for visualisation of HEK293 cells expressing chick HA-Sema5B. Cultures were imaged and the length of the axons from each neuron was measured using ImageJ. For analysis of the function of TAG-1, E4 and E6 DRG were dissociated as above and incubated for 1 h at 37°C in either culture medium alone or in culture medium containing mouse anti-TAG-1 antibody (170 µg/ml, 23.4-5, Hybridoma Bank), and were then added to the appropriate cell culture wells and incubated overnight. The anti-TAG-1 antibody concentration was maintained in the cell cultures for the duration of the experiment.

**Preparation and validation of shRNA vectors**

Sequences for RNAi targeting were analysed using pSico Oligomaker v1.5 software (the Jacks Lab, Massachusetts Institute of Technology, USA) and the oligoduplex palindromes designed for hairpin loop formation were generated by Invitrogen. Two shRNA sequences were generated to target sequences unique to chick Sema5b mRNA: shRNA1 (1203), 5'-GAAATCCCTTTATATA; and shRNA2 (3442), 5'-GGAGTTCAAG-ACACTTTAA. Oligoduplex palindromes were cloned into the XhoI/HpaI restriction sites of the Lentilox 3.7 (pLL3.7) expression vector, which contains an enhanced green fluorescent protein (eGFP) sequence driven by a CMV promoter located downstream of the cloning site (Reynolds et al., 2004). shRNA plasmids were transfected into HEK293 cell lines expressing HA-tagged full-length chick Sema5B or mouse Sema5B using polyethylenimine (Polysciences) as described previously (Browne et al., 2012). The specificity of the shRNA plasmids was verified by its ability to knock down chick Sema5B expression and the lack of knockdown effect on mouse Sema5B expression. This was confirmed by western blot analyses of cell lysates as described previously (Browne et al., 2012).

**In ovo electroporation**

In ovo unilateral electroporation of developing chick spinal cords was performed as previously described (Nakamura and Funahashi, 2001). At the time of electroporation (E3.5/st21 and E5.5/st27), 1 µl purified plasmid DNA...
spinal cord as well as their longitudinal extension, whole mounts of chick
to visualize the extension of afferent axons into the grey matter of the
spinal cord. At st21, electroporation was performed with five 50 ms pulses of
DNA will migrate toward the positive electrode into one side of the developing
formation. Paddle electrodes (CUY650-P3 platinum plate tweezers electrode,
(Invitrogen) were added on top of the embryo to facilitate electric field
experiments. For rescue experiments, equal
DNA constructs were co-transfected (c5B-KD+m5B in Fig. 5). As an
additional control for the rescue experiments to ensure that the lack of
phenotype under the rescue treatment was not due to the dilution of the
shRNA plasmids, injected, shRNA plasmids and an equal volume of a control
pDisplay plasmid were co-transfected (c5B-KD+pDis in Fig. 5). After
electroporation, the openings of the eggs were sealed and the embryos were
allowed to grow further at 38°C until the desired stage for analysis.

Axon-tracing analysis
To visualize the extension of afferent axons into the grey matter of the
spinal cord as well as their longitudinal extension, whole mounts of chick
spinal cord were labelled with the lipophilic tracer DiI as in Schmidt et al.
(2007). Control and shRNA-electroporated spinal cords were dissected with
attached, intact DRG, and fixed in 4% PFA in PBS overnight. Small DiI
crystals were placed against the DRG or against large peripheral nerve
trunks. Spinal cords were left in PBS for 3-4 days before being imaged as
whole mounts with a Leica DM6000CS confocal microscope.

Immunohistochemistry
At the desired stage, the embryonic spinal cords were dissected out into cold
PBS and fixed overnight at 4°C in 4% PFA. On the next day, the spinal
cords were washed in PBS and immered in 15% (v/v) followed by 30% (v/v)
sucrose solutions for cryoprotection. Spinal cords were embedded in O.C.T.
(Sakura Finetek) and cross-sections of 16-30 µm were collected.

The following dilutions were used for labelling: rabbit anti-chick Sema5B
(1:500) (O’Connor et al., 2009) was used to examine Sema5B expression;
mouse anti-TAG-1 (as above; 1:500); rabbit anti-TrkC (1:1500, Cell
Signaling, #3376); rabbit anti-HA (as above; 1:500); rabbit anti-Pax6
(1:500, Hybridoma Bank), mouse anti-IslaI (1:500, Hybridoma Bank) and
mouse anti-Nkx2.2 (1:500, Hybridoma Bank). Immunolabelling was
visualised with a Leica confocal microscope. Abrantly projecting axons
were counted per section and 5-15 sections were quantified per animal. The
average number of aberrant collaterals per section was calculated and
averaged across the number of animals (n= 4-10 chicks per treatment).

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Competing interests
The authors declare no competing financial interests

Author contributions
R.O.L. performed the majority of experiments, analysed the data and prepared the
manuscript. W.W. prepared the shRNA constructs and confirmed their knockdown of
Sema5B in heterologous cells and contributed to manuscript preparation.
A.L. performed in situ hybridisation and immunocytochemistry on developing spinal
cords. J.A. generated in situ hybridisation probes. T.P.O. developed the
experimental concepts, supervised experiments, wrote and edited the manuscript.

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Supplementary material
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